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Cloning and characterization of a novel human phosphatidic acid phosphatase type 2, *PAP2d*, with two different transcripts *PAP2d_v1* and *PAP2d_v2**

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Abstract

This study reports the cloning and characterization of a novel human phosphatidic acid phosphatase type 2 isoform cDNAs (*PAP2d*) from the foetal brain cDNA library. The *PAP2d* gene is localized on chromosome 1p21.3. It contains six exons and spans 112 kb of the genomic DNA. By large-scale cDNA sequencing we found two splice variants of *PAP2d, PAP2d_v1* and *PAP2d_v2*. The *PAP2d_v1* cDNA is 1722 bp in length and spans an open reading frame from nucleotide 56 to 1021, encoding a 321aa protein. The *PAP2d_v2* cDNA is 1707 bp in length encoding a 316aa protein from nucleotide 56–1006. The *PAP2d_v1* cDNA is 15 bp longer than the *PAP2d_v2* cDNA in the terminal of the fifth exon and it creates different ORF. Both of the proteins contain a well-conserved PAP2 motif. The *PAP2d_v1* is mainly expressed in human brain, lung, kidney, testis and colon, while *PAP2d_v2* is restricted to human placenta, skeletal muscle, and kidney. The two splice variants are co-expressed only in kidney. (Mol Cell Biochem **272:** 91–96, 2005)

Key words: expression pattern, *PAP2d*, PAP2 motif, RT-PCR, splice variant

Introduction

Phosphatidic acid phosphatases (PAPs) are a family of integral membrane glycoproteins. The enzyme activity of PAP could be separated into two components, type 1 (PAP1) and type 2 (PAP2) [1, 2]. PAP1 was defined as a cytosolic/endoplasmic reticulum-localized activity that was both dependent on Mg^{2+} and inactivated by the thio-reactive compound *N*-ethylmaleimide (NEM) [3, 4]. This activity shows a pronounced selectivity for PA and appears to be a soluble enzyme that can reversibly associate with cellular membranes [5]. PAP1 was reported to translocate to microsomes on stimulation of triacylglyceride synthesis, while PAP-2 was unaffected by lipid metabolism status [1].

Conversely, a Mg^{2+} dependent, NEM insensitive membrane-associated activity was classified as PAP-2 [4, 5]. Type-2 phosphatidic acid phosphatases (PAP2) catalyze the hydrolysis of phosphatidic acid (PA) to diacylglycerol (DG).

*The nucleotide sequences reported in this paper have been submitted to GenBank under accession number: AY574039 (*PAP2d_v1*) and AY634620 (*PAP2d_v2*).

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They also catalyze the dephosphorylation of a number of bioactive lipid mediators including the bioactive signaling molecules lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and ceramide 1-phosphate (Cer1P) [6, 7]. These mediators exert complex effects on cell-function through both actions at cell-surface receptors and on intracellular targets. The PAP-catalyzed dephosphorylation of these substrates can both terminate their signaling actions and itself generate further molecules with biological activity [4]. These are widely expressed proteins with homologs in many organisms including mammals, bacteria, yeast, and Drosophila [6]. Unlike the soluble PAP1 enzymes, PAP2 enzymes are integral membrane proteins and only the type 2 activity is decreased in transformed fibroblasts [8]. Therefore, it was supposed that PAP1 is involved in phospholipid metabolism while PAP2 is involved in signal transduction.

Three human isoforms of membrane associated phosphatidic acid phosphatase have been described (hPAP 2a, 2b and 2c) and the three enzymes have been shown to have broad substrate specificity and wide tissue distribution [1]. PAP 2d is the fourth member of the family of PAP2 isoenzymes. This enzyme shares the putative transmembrane topology of the other PAP2 isoenzymes, including six regions of predominantly hydrophobic amino acids linked by extramembrane regions [6]. This proposed transmembrane structure is similar to that of the membrane-spanning portions of membrane-bound adenylyl cyclases and the transport proteins of the P-glycoprotein superfamily, which consist of a short N terminus and two transmembrane regions consisting of six hydrophobic spans that link globular cytoplasmic domains [9]. Comparison of the sequence of h PAP2 with other phosphatases defines a protein motif composed of three regions of conserved sequence. PAP2 homologs have been identified in yeast and Drosophila [10, 11].

During the large-scale sequencing analysis of a human fetal brain cDNA library, we isolated a novel cDNA *PAP2d* and found two splice variants *PAP2d_v1* and *PAP2d_v2*. The *PAP2d_v1* cDNA has 15 bp longer than the *PAP2d_v2* cDNA in the terminal of the fifth exon and it makes the two splice variants encode different ORFs. As both of the proteins contain the well-conserved PAP2 motif, we termed the novel genes phosphatidic acid phosphatase type 2d (*PAP2d*). Here, we report the cloning and initial characterization of *PAP2d_v1 and PAP2d_v2*, together with data of their gene structures and mRNA tissue distributions.

Materials and methods

cDNA library construction

A cDNA library was constructed in a modified pBluescript II SK (+) vector with human foetal brain mRNA purchased from Clontech. The modified vector was constructed by introducing 2 *Sfi*I recognition sites, i.e. *Sfi*I A (5′-GGCCA TTATGGCC-3′) and *Sfi*I B (5′-GGCCGCCTCGGCC-3′), between the *Eco*RI and *Not*I sites of pBluescriptII SK (+) (Stratagene). Double-stranded cDNAs were synthesized using SMART™ cDNA Library Construction Kit (Clontech) following manufacturer's instructions. After *Sfi*I digestion and cDNA size fractionation, cDNAs longer than 500 bp were ligated into the *Sfi*I A and *Sfi*I B sites of the modified pBluescriptII SK (+) vector. The constructs were then transformed into *E. coli* DH5α cells. Individual clones were cultured overnight in LB broth with 100 μg/ml ampicillin, and plasmids were isolated using QIAwell plasmid purification system (QIAGEN).

Sequencing of the cDNA inserts

The cDNA inserts were sequenced on an ABI PRISM™ 377 DNA sequencer (Perkin–Elmer) using the BigDye Terminator Cycle Sequencing Kit and BigDye Primer Cycle Sequencing Kit (Perkin–Elmer) with-21M13 primer, M13Rev primer and synthetic internal walking-primers designed according to the obtained cDNA sequence fragments. Each part of the insert was sequenced at least three times bi-directionally. Subsequent editing and assembly of all the sequences from one clone was performed using Acembly (Sanger's Center).

Bioinformatics analysis

DNA sequence homology searches and comparisons were performed using BLAST-N and BLAST-X at the National Center for Biotechnology Information (NCBI) network service (http://www.ncbi.nlm.nih.gov/blast). BLAST-N search against the human genome was performed to identify the chromosomal localization and the gene structure of this gene. The predicted amino acid sequence of human NRF cDNA was compared against the profile entries to find the occurrence of known profiles (http://www.expasy.ch/pfscan). Multiple sequences alignments were performed by GeneDoc programme (http://www.psc.edu/biomed/genedoc/). Other database from GenBank, Swiss Pro, PDB and EXPASY were also used. Relative analysis softwares include Gene Runner, Primer Premier5.0.

Assessment of human NRF mRNA tissue distribution

Human Multiple Tissue cDNA (MTC) panels (Clontech) were used as PCR templates according to the manufacturer's protocol. PCR were performed using Taq Plus polymerase

(Sangon) at the following programme: 0.5 min at 94 °C, 0.5 min at 62 °C, 30 s at 72 °C. The reaction cycles for *PAP2d* and G3PDH (as control) are 40 and 24, respectively. The PCR products were then electrophoresed on a 2.5% agarose gel. PCR primers for *PAP2d* are indicated from 5′ to 3′ as the following: sense, GCAGAAAATGAGCATATACACATGG; antisense, TCATGTGACTTCTGCGAAGGCAG. The region between sense primer and antisense primer spans 132 bp in the *PAP2d_v1* and 117 bp in the *PAP2d_v2*.

Results and discussion

Identification and sequence analysis of human PAP2d cDNA

A novel human phosphatidic acid phosphatase type 2 isoform encoding gene *PAP2d* was obtained from the foetal brain cDNA library. By large-scale cDNA sequencing we found two different splice variants of *PAP2d*. So we termed them *PAP2d_v1* and *PAP2d_v2*. The *PAP2d_v1* cDNA is 1722 bp in length and spans an open reading frame from nucleotide 56 to 1021, encoding a putative 321 residue protein. The *PAP2d_v2* cNDA is 1707 bp in length encoding a 316aa polypeptide from nucleotide 56 to 1006 (Fig. 1). The *PAP2d_v1* cDNA is 15 bp longer than the *PAP2d_v2* cDNA in the terminal of the fifth exon which leads to different ORF. The predicted molecular mass of the two proteins are both 35 kDa and the isoelectric point is 6.87. Human genomic blast showed that the cDNA sequences were mapped to chromosome 1p21.3. It spanned more than 112 Kb of the genomic DNA and consisted of six exons (Fig. 2). All the sequence features of the exon–intron junctions are consistent with the AG-GT rule (Table 1).

Although there is no in-frame stop codon, the cDNA sequence is still considered to be full length because there is partial sequence of the promoter in about 200 bp upstream of the 5′ end of our cDNA (http://www.fruitfly.org/seq_tools/promoter.html; upstream sequence from htgs database), and there is not any ATG codon between the promoter region and the translational start codon. In addition the possible polyadenylation signals AATAAA were found near the 3′-end in both the nucleotide sequences. All these sequence features indicate that the two novel cDNAs are full-length.

Homologous analysis shows that the novel cDNAs belong to the human PAP2 superfamily. So we termed them phosphatidic acid phosphatase type 2d (*PAP2d*) (The GenBank accession numbers for *PAP2d_v1* and *PAP2d_v2* are AY574039 and AY634620).

Multiple alignment of the deduced amino acid sequences of hPAP2a, 2b, 2c, and 2d was performed. Hydropathy analysis of all the five sequences suggests that PAP2a, 2b, 2c and 2d are integral membrane proteins with six membrane-spanning

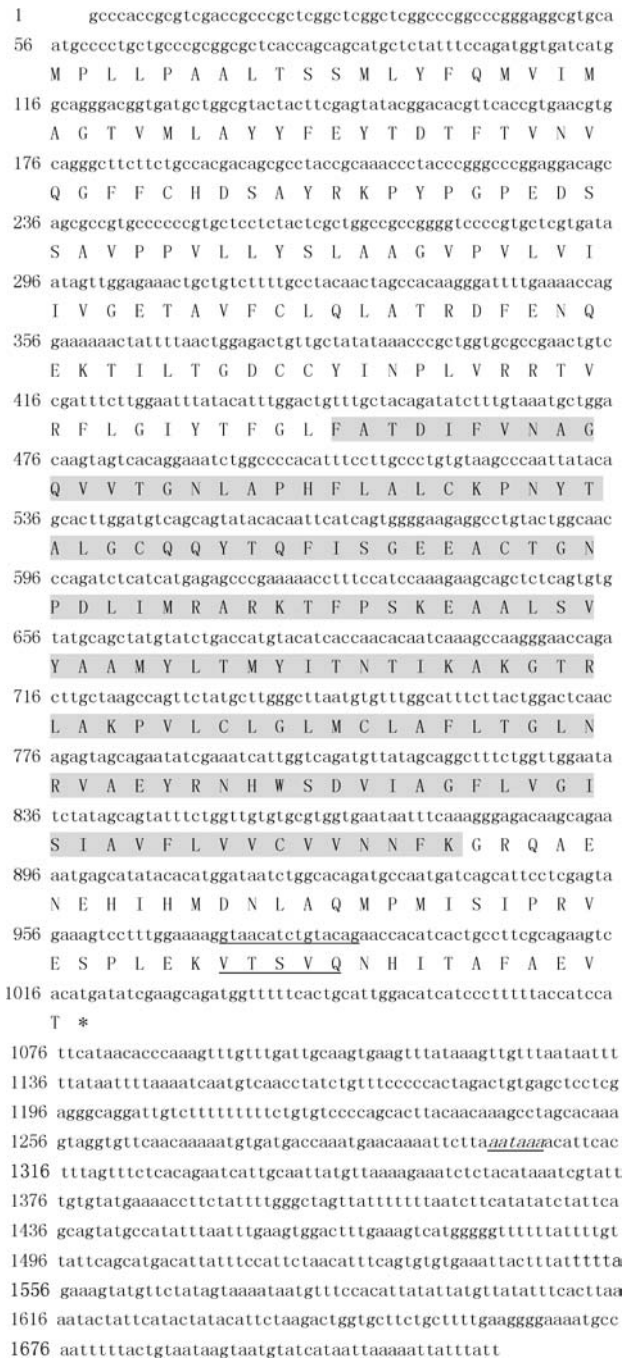

Fig. 1. The nucleotide sequence and deduced amino acid sequence of the *PAP2d_v1* gene. The conserved PAP2d are shaded. The possible polyadenylation signals *(AATAAA)* are in italic. The 15 bp nucleotides and the five amino acids not presented in *PAP2d_v2* are underlined.

regions of 17–25 hydrophobic amino acid residues. These sequences are underlined in Fig. 3a and denoted as regions I–VI in Fig. 3b. Comparison of the five sequences identifies three regions of conserved sequences (denoted as A, B, and C in Fig. 3), which contain invariant amino acids that define a

Table 1. Exon–intron analysis of *PAP2d_v1* and *PAP2d_v2* genes

Exon				Intron	
Number	Length (bp)	Splicing acceptor	Splicing donor	Number	Length (bp)
		PAP2d_v1			
1	292		CGTGCTCGTG***gt***aagcccgg		
2	133	ttctttca***ag***ATAATAGTTG	CGATTTCTTG***gt***atgtgttc	1	47693
3	251	tcttcccc***ag***GAATTTATAC	GTATCTGACC***gt***aagtaagg	2	3287
4	177	aatgtttc***ag***ATGTACATCA	AGTATTTCTG***gt***aagtacaa	3	31011
5	135	gaaattttt***ag***GTTGTGTGCG	ATCTGTACAG***gt***gactcaca	4	6961
6	734	ctttttgc***ag***AACCACATCA		5	21681
		PAP2d_v2			
1	292		CGTGCTCGTG***gt***aagcccgg		
2	133	ttctttca***ag***ATAATAGTTG	CGATTTCTTG***gt***atgtgttc	1	47693
3	251	tcttcccc***ag***GAATTTATAC	GTATCTGACC***gt***aagtaagg	2	3287
4	177	aatgtttc***ag***ATGTACATCA	AGTATTTCTG***gt***aagtacaa	3	31011
5	120	gaaattttt***ag***GTTGTGTGCG	TTTGGAAAAG***gt***aacatctg	4	6961
6	734	ctttttgc***ag***AACCACATCA		5	21696

Note. Intron and exon nucleotide sequence are shown in lower-case and upper-case letters, respectively. Bold italics lettering indicates donor and acceptor splice site.

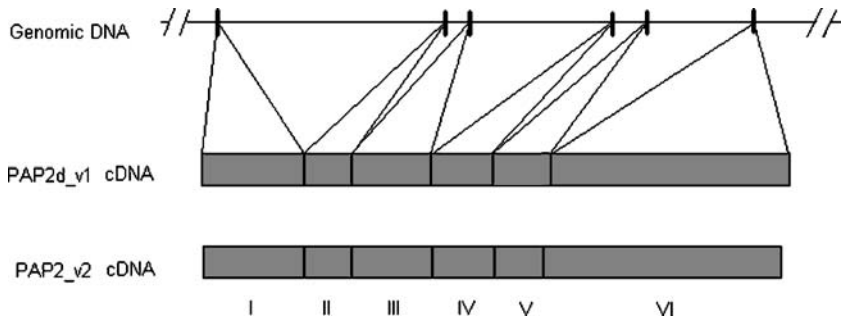

Fig. 2. Human genomic localization of cDNA sequence for two splice variants. The *PAP2d_v1* and *PAP2d_v2* gene both consist of six exons. *PAP2d_v1* is 15 bp longer than *PAP2d_v2* from 973—988 bp in the terminal of the fifth exon. The symbols (I, II, etc.) shown in the bottom of the figure represent the exons I to VI of *PAP2d*. The white in the box of *PAP2d_v1* represents the nucleotides longer than *PAP2d_v2*.

signature sequence motif shared by several proven or putative lipid phosphatases, the mammalian glucose 6-phosphatases, and some bacterial nonspecific and acid phosphatases. These conserved regions lie predominantly within the hydrophilic regions of the proteins, and homologous sequences are also found in a group of soluble globular proteins including bacterial acid phosphatase, mammalian glucose 6-phosphatase, and fungal vanadium-dependent chloroperoxidase. It is notable that in comparison with PAP2a, 2c and 2d, PAP2b contains an extended N-terminus with an enrichment of basic amino acid residues. The 40 amino acids of the c-terminus of the five proteins are also highly divergent. All of them contain a single consensus site for N-linked glycosylation (residue 164 in PAP2d).

Expression pattern of the two splice variants

In order to display the tissue distribution of the two splice variants *PAP2d_v1* and *PAP2d_v2*, RT-PCR analysis was performed with the multiple tissue cDNA (MTC) panel kit (Clontech). The expression pattern was shown in Fig. 4. The results showed that the size of the amplification bands was in accord with the expected 132 bp and 117 bp of the two splice variants and the results were also confirmed by DNA sequencing. The *PAP2d_v1* is mainly expressed in human brain, lung, kidney, testis and colon while *PAP2d_v2* is restricted to human placenta, skeletal muscle, and kidney (Fig. 4). The two splice variants only co-expressed in kidney. It is a hypothesis that the expression of these two splice variants is

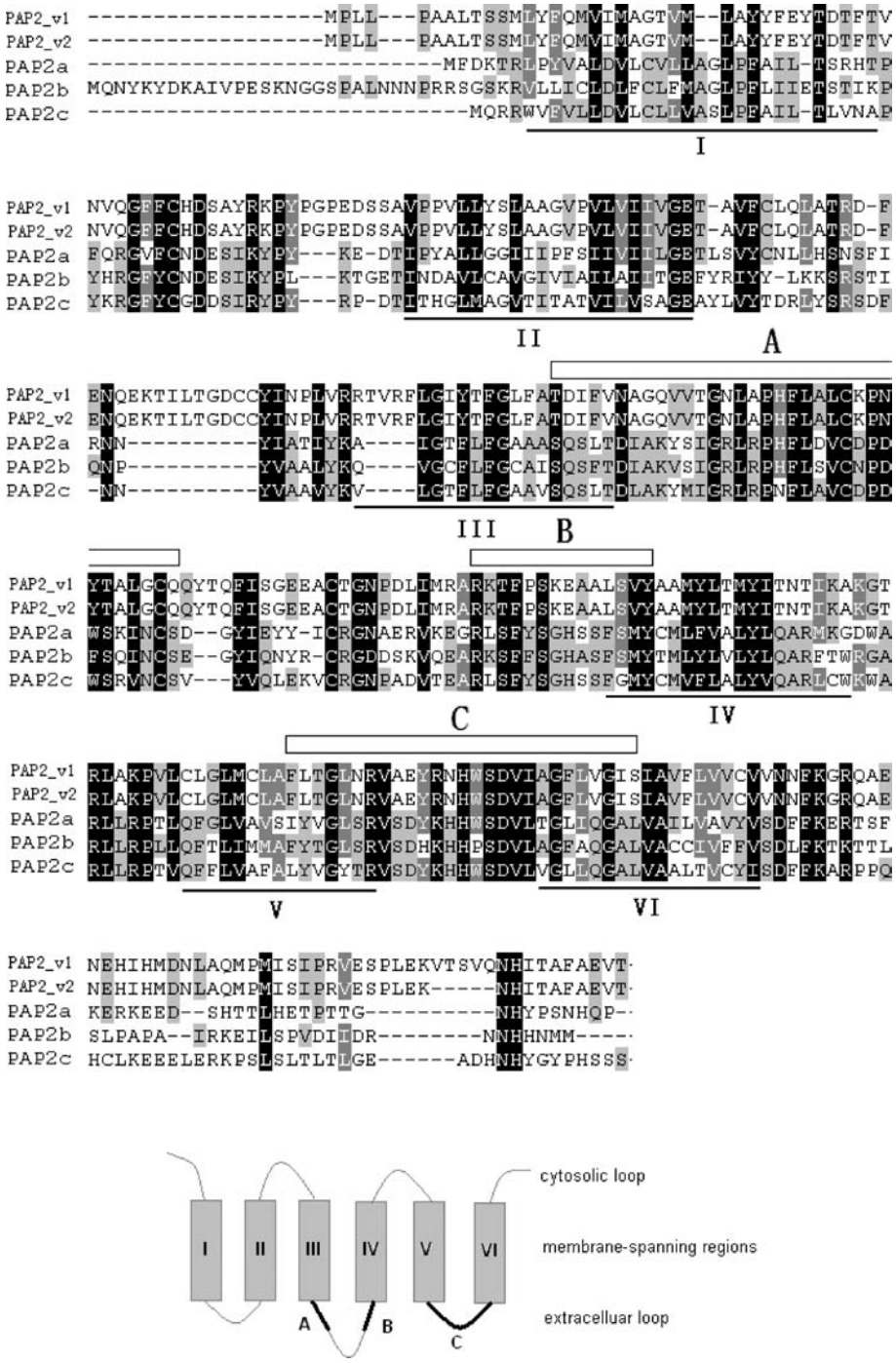

Fig. 3. Sequence comparison of PAP2a, 2b, 2c and 2d. The alignments were performed by GeneDoc programme (http://www.psc.edu/biomed/genedoc/): Black (100% similarity); grey (80–90% similarity); light grey (60–70% similarity); Boxed regions denoted A, B, and C encompass regions of amino acid similarity that are conserved among other phosphatases as described under "Result and Discussion." Regions of hydrophobic amino acids denoted I–VI are underlined. The lower part of the figure is the predicted transmembrane topology of the proteins.

regulated by the same mechanism and they possibly played the common biological roles. PAP2 function in phospholipids metabolism by catalyzing the dephosphorylation of a number of bioactive lipid mediators including lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) and phosphatidic acid (PA). These mediators exert complex effects on cell function through both actions at cell surface receptors and on intracellular targets. PA has been proposed to play an essential

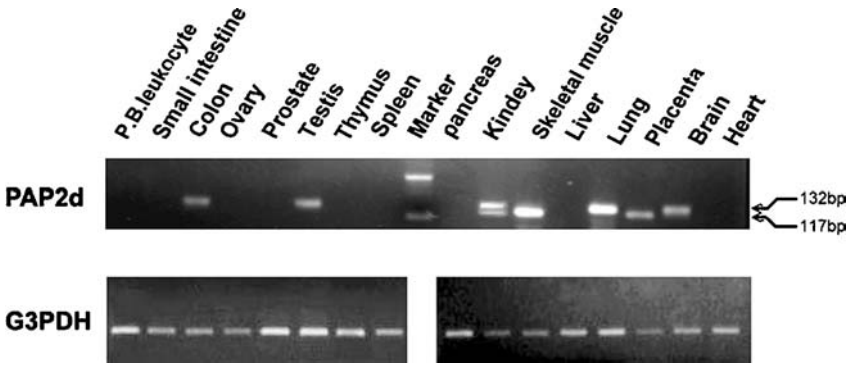

Fig. 4. Tissue distribution of human PAP2d mRNA. Reverse transcription-PCR analysis of human cDNAs for human PAP2d and G3PDH (as a control). Prenormalized cDNAs from sixteen human adult tissues were purchased from CLONTECH and employed as templates in PCR reactions containing human PAP2d-specific primers described in "Materials and Methods". All the PCR products were run on 2.5% agarose gel. In the marker line the above and bottom bands are 250 bp and 100 bp. The amplification bands of 132 bp and 117 bp are the *PAP2d_v1* and *PAP2d_v2* gene, respectively.

role in recruitment of cytosolic coatomer complexes to the surfaces of the respective membrane compartments involved in these transport processes, which in turn initiates formation of coated transport vesiclesp [12]. Such a mechanism would require tight control of PA levels, and localization of PAP2 activity to this membrane compartment may therefore play an important role in this process. Additionally, many of the products of PAP2-mediated hydrolysis (diacylglycerol, sphingosine, and ceramide) are themselves important signaling molecules [13]. Due to their apparent broad substrate specificity, PAP2 is poised to regulate several signaling pathways by simultaneously creating and destroying bioactive lipids. Clearly, establishment of the functions carried by the PAP2 enzymes in cellular lipid metabolism remains an important priority.

In conclusion, we have identified a novel human *PAP2d* cDNA besides *PAP2a, 2b* and *2c* and it has two splice variants, *PAP2d_v1* and *PAP2d_v1*. The tissue distribution, genomic organization and chromosome location of *PAP2d* were determined. Expression pattern indicates that the two genes are expressed in different tissues except in kidney. Further research is required to determine the substrate of the PAP2d and the distinct biological functions of the two novel proteins.

Acknowledgments

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